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Method and Means for Producing a Fibrinogen Binding Protein and its Use in Biotechnology.

The invention relates to the field of gene technology and is concerned with recombinant DNA molecules, which contain a nucleotide sequence coding for a protein or polypeptide having fibrinogen-binding activity. Moreover the invention comprises microorganisms (including viruses) containing the aforesaid molecules, and the use thereof in the production of the aforesaid protein or polypeptide and their use in biotechnology.

During the last decay the coagulase-negative staphylococci (CNS) have attracted an increasing attention. Along with the development of human and veterinary medicine the number of susceptible hosts have increased. Advanced surgery, an increased use of biomaterial, medication with cytostatic, antibiotics and other drugs together with an increased frequency of antibiotic resistant strains of CNS have incresed the susceptibility of the host. Concerning the veterinary importance of the CNS it is known that they e.g. can cause both subclinical and clinical inflammation in the bovine udder. The existence of bacteria that bind specifically to fibrinogen has been known for many years. The role of fibrinogen binding in the interaction process between the host and *Staphylococcus aureus* is still not clear but the fibrinogen-binding has been considered as one potential virulence factor of this species for instance in endocarditis (Moreillon et al 1995). Concerning CNS they have not been reported to bind fibrinogen (Wadström and Rozgony, 1986) and no protein with fibrinogen binding properties has to our knowledge been described originating from CNS. However the present invention describes the isolation and characterization of such protein using gene cloning. Furthermore the invention describes different methods to measure the fibrinogen binding activity on cells of CNS and the use of this protein in biotechnology.

Generally it might be difficult to obtain a homogenious and a reproducible product if such a binding protein was prepared from staphylococcal cells directly. Moreover staphylococci are pathogenic and need complex culture media which involves

complications in large-scale cultur s. There is thus a need for a new method for producing a fibrinogen binding protein (or fragments thereof).

The present invention relates to a recombinant DNA molecule comprising a nucleotide sequence which codes for a protein or polypeptide having fibrinogen-binding activity. The natural source of this nucleotide sequence is of course the *S. epidermidis* strain HB but with the knowledge of the nucleotide and deduced amino acid sequence presented here, the gene or parts of the gene can be isolated or made synthetically. In particular the knowledge of the deduced amino acid sequence for the part of the protein responsible for the fibrinogen binding activity can be used to produce syntethic polypeptides, which retain or inhibit the fibrinogen binding. These polypeptides can b labelled with various compounds such as enzymes, fluorescence, biotin (or derivatives of), radioactivity, etc and used for e.i. in diagnostic tests such as ELISA- or RIA-techniques.

For production of a recombinant DNA molecule according to the invention a suitable cloning vehicle or vector, for example a phagemid, plasmid or phage DNA, may be cleaved with the aid of a restriction enzyme whereupon the DNA sequence coding for the desired protein or polypeptide is inserted into the cleavage site to form the recombinant DNA molecule. This general procedure is known per se, and various techniques for cleaving and ligating DNA sequences have been described in the literature (see for instance US 4,237,224; Ausubel et al 1991; Sambrook et al 1989), but to our knowledge these techniques have not been used for the present purpose. If the *S. epidermidis* strain HB is used as the source of the desired nucleotide sequence it is possible to isolate said sequence and to introduce it into a suitable vector in manner such as described in the experimental part below or, since the nucleotide sequence is presented here, use a polymerase chain reaction (PCR)-technique to obtain the complete or fragments of the fig gene.

Hosts that may be used are, microorganisms (which can be made to produce the protein or active fragments thereof), which may comprise bacterial hosts such as strains of e.g. *Escherichia*

coli, *Bacillus subtilis*, *Staphylococcus* sp., *Lactobacillus* sp. and furthermore yeasts and other eucaryotic cells in culture. To obtain maximum expression, regulatory elements such as promoters and ribosome binding sequences may be varied in a manner known per se. The protein or active peptide thereof can be produced intra- or extracellularly. To obtain good secretion in various bacterial systems different signal peptides could be used. To facilitate purification and/or detection the protein or fragment thereof could be fused to an affinity handle and /or enzyme. This can be done on both genetic and protein level. To modify the features of the protein or polypeptide thereof the gene or parts of the gene can be modified using e.g. *in vitro* mutagenesis; or by fusion of other nucleotide sequences that encode polypeptides resulting in a fusion protein with new features.

The invention thus comprises recombinant DNA molecules containing a nucleotide sequence which codes for a protein or polypeptide having fibrinogen-binding properties. Furthermore the invention comprises vectors such as e.g. plasmids and phages containing such a nucleotide sequence, and organisms, especially bacteria as e.g. strains of *E.coli*, *B.subtilis* and *Staphylococcus* sp., into which such a vector has been introduced. Alternatively, such a nucleotide sequence may be integrated into the natural gene material of the microorganism.

The application furthermore relates to methods for production of a protein or polypeptide having the fibrinogen binding activity of protein FIG or active fragments thereof. According to this method, a microorganism as set forth above is cultured in a suitable medium, whereupon the resultant product is isolated by some separating method, for example ion exchange chromatography or by means of affinity chromatography with the aid of fibrinogen bound to an insoluble carrier.

Vectors, especially plasmids, which contain the protein FIG encoding nucleotide sequence or parts thereof may advantageously be provided with a readily cleavable restriction site by means of which a nucleotide sequence, that codes for another product, can be fused to the protein FIG encoding nucleotide sequence, in order to express a so called fusion protein. The fusion protein

may be isolated by a procedure utilizing its capacity of binding to serum albumin and/ or IgG or to oth r fusion partners like histidine residues, glutathione S transferase etc, whereupon the other component of the system may if desired be liberated from the fusion protein. This technique has been described at length in WO 84/03103 in respect of the protein A system and is applicable also in the present context in an analogous manner. The fusion strategy may also be used to modify, increase or change the fibrinogen binding activity of protein FIG (or part thereof) by fusion of other fibrinogen binding molecules.
The present invention also apply to the field of biotechnology that concerns the use of bacterial cell surface components as immunogens for vaccination against CNS infections.

Starting materials

Bacterial strains, phages and cloning vectors

Staphylococcus epidermidis strain HB was obtained from Dr Åsa Ljungh , Lund, Sweden.
E.coli strain TG1 and strain MC1061 were used as bacterial host for construction of the library and production of the phage stocks. The *E. coli* phage R408 (Promega, Madison, WI, USA) was used as helper phage.
The phagemid vector pG6H6 used is described Jacobsson and Frykberg (1996).
All strains and plasmid- or phagemid- constructs used in the examples are available at the Department of Microbiology at the Swedish University of Agricultural Sciences, Uppsala, Sweden.

Buffers and media

E. coli was grown on LB (Luria Bertani broth) agar plates or in LB broth (Sambrook et al 1989) at 37°C. In appropriate cases the LB medium was supplemented with glucose to a final conc. of 2%. Ampicillin was in appropriate cases added to the *E. coli* growth media to a final conc. of 50 µg/ml. Staphylococci were grown at 37°C on bloodagar-plates (containing 5% final conc. bovine blood) or in Tryptone Soya Broth (TSB obtained from Oxoid. Ltd

Basingstoke, Hants., England) PBS: 0,05M sodium phosphate pH 7.1, 0.9 % NaCl. PBS-T: PBS supplemented with TWEEN 20 to a final conc. of 0.05 %.

Preparation of DNA from staphylococci and streptococci.
Strains of *S. epidermidis* or *S. aureus* were grown overnight in TSB. Next morning the cells were harvested and the chromosomal DNA prepared according to Löfdahl et al (1983). Chromosomal DNA from streptocci have earlier been described in WO 95/07300.

Proteins and other reagents
Human fibrinogen was obtained from (IMCO Ltd, Stockholm, Sweden). Human serum albumin (HSA), fibronectin, IgA, lactoferrin and transferrin were obtained from Sigma, St. Louis, USA). Bovine serum albumin (fraction V, ria grade) was obtained from USB (cat. no.10868). $\alpha_2$macroglobulin (α_2M) and collagen type I were obtained from Boehringer, Mannheim, Germany). Vitronectin was obtained from Bional, Tartu, Estonia and human IgG from Kabi, Stockholm, Sweden. Elastin was obtained from ICN Pharmaceuticals Inc. CA, USA and pepsin from KEBO lab Stockholm, Sweden.
DNA probes were labelled with α^{32}P-ATP by a random-priming method (Multiprime DNA labelling system; Amersham Inc. Amersham, England)
Nitrocellulose(NC)-filters(Schleicher&Schüll,Dassel,Germany) were used to bind DNA in hybridization experiments or proteins in Western-blot techniques.
In order to analyze protein samples by native or sodium dodecyl sulphate -polyacrylamid gel electrophoresis (SDS-PAGE) the PHAST-system obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden was used according to the suppliers recommendations.
Oligonucleotides used were sythesized by (Pharmacia, Uppsala, Sweden).
Micro Well plates (MaxiSorp, Nunc, Copenhagen, Denmark) were used in panning experiment. Plasmid DNA was prepared using Wizard Minipreps (Promega) and the sequence of the inserts was determined as descibed by Jacobsson and Frykberg (1995). The sequences obtained were analysed using the PC-gene program

(Intelligenetics, Mountain View, CA, USA)

Routine methods

Methods used routinely in molecular biology are not described such as restriction of DNA with endonucleases, ligation of DNA fragments, plasmid purification etc since these methods can be found in commonly used manuals (Sambrook et al 1989, Ausubel et al 1991). Ligation reactions were performed using Ready-To-Go T4 DNA Ligase (Pharmacia, Uppsala, Sweden). For polymerase chain reaction amplification the Gene Amp™ kit, obtained from Perkin Elmer Cetus, was used. Sequence reactions were performed using "Sequenase, version 2.0" kit (United States Biochemical Corporation, Cleveland, Ohio, USA). Alternatively the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit was used and the samples analysed using the Applied Biosystems 373A DNA Sequencer.

Example 1. The adherence of *Staphylococcus epidermidis* to immobilized fibrinogen and investigation of the nature of the binding mechanism (A-F).

Strains of *Staphylococcus epidermidis* isolated from cases of peritonitis were grown on Blood agar plates at 37°C overnight. The bacteria from one plate was harvested with 5 ml phosphate buffered saline (PBS), washed once, and the optical density (OD) was adjusted to 1.0.

(A) Bacterial adherence. Fibrinogen was dissolved in PBS at 10 mg/ml and added in serial 3-fold dilution to microtiter wells (Nunc), from top to bottom. The plates were incubated overnight at room temperature (RT). To cover uncoated plastic sites the plates were coated with 1% bovine serum albumin for 1 hour at 37°C. The plates were washed with PBS with 0.05% Tween 20 (PBST). Next, bacteria were added in serial 2- fold dilution in PBST, from left to right, to the fibrinogen coated microtiter plates. Bacterial adherence was allowed for 2 hours at 37°C or at 4°C overnight. Non-adherent bacteria were washed off and the bound bacteria were airdried. The crosswise dilution of both fibrinogen and bacteria allows estimation of bacterial binding

both as a function of fibrinogen concentration and of amount of bacteria. Determination of bacterial adherence was done by optical reading using a microtiter plate reader at A 405. The turbidity and light scatter caused by bound bacteria results in a reading ranging from 0.00 to 0.20. An example of adherence values as a function of fibrinogen coating concentration is shown in Figure 1 for three different strains (2,19 and JW27). These conditions for adherence determination were used in the following experiments.

(B) Adherence blocking by antibodies against fibrinogen. In a modification of the experiment performed above, antibodies against fibrinogen (anti Fg) (Sigma) were added 1 hour prior to addition of bacteria (OD=1.0) to the immobilized fibrinogen. As a control, antibodies against fibronectin (anti Fn) (Sigma) were added in a separate experiment. Figure 2 shows that antibodies against fibrinogen (circles) inhibited adherence better than antibodies against fibronectin could (squares). The mean values and standard errors from three separate experiments are shown.

(C) Adherence blocking by soluble fibrinogen. Soluble fibrinogen was added to the bacteria at concentrations indicated in Figure 3 and incubated for 1 hour at 37°C before addition to plates coated with fibrinogen as described above. Adherence of *S. epidermidis* strain 19 (filled circles) was inhibited to around 30%. As a control, inhibition of *Staphylococcus aureus* strain Newman was measured in a similar experimental setup (open circles). Mean values and standard errors from three separate experiments are shown. Although significant inhibition of adherence of *S. epidermidis* was obtained, inhibition of *S. aureus* was more pronounced.

(D) Reduction of binding after protease treatment of bacteria. Bacteria were treated for 30 minutes at 37°C with protease K, at concentrations indicated in Figure 4, prior to addition to immobilized fibrinogen. Protease treated bacteria were extensively washed after protease treatment to avoid protease digestion of the immobilized fibrinogen. Four different strains of *S. epidermidis* (2, 19, 269 and HB) and *S. aureus* (strain Newman) were used in this experiment. All strains tested showed

sensitivity to prot as treatment, thus the adherence to fibrinogen depends on a surf ce protein.

(E) Adherence blocking by LiCl extract of *S. epidermidis*. *S. epidermidis* cells, grown and harvested as described above, were treated with 1M LiCl at 40°C for 2 hours with continous gentle stirring. The bacteria were centrifuged and the bacteria-fre supernatant was filtered and dialysed against PBS. Surface associated proteins bound to the cells by hydrophobic interactions are thereby released. This LiCl extract, presumably containing a fibrinogen binding protein, was used to inhibit adherence of *S. epidermidis* to immobilized fibrinogen in the following way: LiCl extract at various dilutions was added to the immobilized fibrinogen and incubated for 1 hour at 37°C. The plates were washed and bacteria added for adhesion testing. Figure 5 shows that adherence was better the more the LiCl extract was diluted; i.e. an adhesion-inhibitory compound is present in the LiCl extract.

Example 2. Isolation of a clone expressing fibrinogen binding activity.

A gene library of *S. epidermidis* strain HB was produced in a manner as described by Jacobsson and Frykberg (1996). Staphylococcal DNA was randomly fragmented by sonication The library resulted in $4x10^7$ independent clones, which after amplification had a titer of $2x10^{12}$ cfu/ml. Two hundred microliters of the library were added to each of three wells and incubated for 4 hour at room tempereature (RT). The wells were washed extensively with PBS-T and once with 50mM Na-citrate/140 mM NaCl, pH 5.4. Finally, the bound phage were eluted stepwise in the same buffer with decreasing pH (3.4 and 1.8). The eluates from the three wells were neutralized with 2 M Tris-HCl, pH 8.6. Aliquots of the eluates were used to infect *E. coli* TG1 cells, which then were grown overnight on LA plates containing glucose and ampicillin. The colonies (obtained after infection of TG1 cells with the phage and eluted at pH 3.4 and 1.8 in the primary panning) were collected by resuspension in LB medium and infected with helper phage R408 (10^{12} plaque-forming units (pfu)) for

production of enriched phage stocks. Thereafter, the infected bacteria were mixed with 4 ml 0.5% soft agar and poured on one LA plate with ampicillin. After incubation over night 37°C the phages were collected as described by Jacobsson and Frykberg (1996). The resulting phage stock was repanned against fibrinogen as described above. The result presented in Table 1. shows that there is an enrichment of clones having affinity to fibrinogen.

Table 1.

Panning	*Ligand* Fibrinogen	IgG
1st		
Wash	1.6×10^3 cfu/ml	-
pH 5.4	1.6×10^3 cfu/ml	-
pH 3.4	2.1×10^3 cfu/ml	-
pH 1.8	7.0×10^3 cfu/ml	-
2nd		
Wash	1.2×10^3 cfu/ml	2.2×10^3 cfu/ml
pH 5.4	4.4×10^3 cfu/ml	6.2×10^3 cfu/ml
pH 3.4	4.3×10^4 cfu/ml	1.4×10^3 cfu/ml
pH 1.8	2.0×10^3 cfu/ml	8.0×10^3 cfu/ml

Example 3. DNA sequencing and sequence analysis.

Eigth colonies coming from the 2nd panning (pH 3.4) against fibrinogen described in Example 2 were chosen for further studies. Phagemid DNA from these colonies were prepared and partially sequenced. Seven of the clones seemed to contain the same insert. One of these seven clones called pSE100 was chosen for further studies. Purfied phagemic DNA from the clone pSE100 was analysed by restriction mapping which revealed that the phagemid contained an insert of ~1.8 kilo base pair (kb). The nucleotide (nt) sequences of the complete inserts of pSE100 was determined and the nt and deduced amino acid (aa) sequences were analysed using the PC-gene program. This analysis revealed that the insert of pSE100 contains an open reading frame of 1.745 nt (sequence list). Thus the insert encodes a 581 aa protein, termed protein FIG (and th corresponding gene termed *fig*), with

a calculated molecular mass of ~65 kDa (sequence list). Furthermore, the sequence analysis show that the insert of pSE100 is in the correct reading frame with the vector sequences in the 5'-and 3'-ends. This means that the insert gives rise to a fusion with the *pel* leader and the *myc* tail (sequence list) and that the native 5'- and 3'-ends of the *fig* gene is not present in the pSE100 clone. Interestingly, sequence analysis of the deduced amino acid squence of the insert reveals similarities to one potential virulence factor of *S. aureus* called clumping factor (McDevitt et al 1994).

Example 4. Properties of the fibrinogen binding protein encoded from pSE100.

A) Specificity of the fibrinogen binding. The phagemid pSE100 was elctroporated into competent E.coli TG1 cells. After growth over night on a LA plate (containing ampicillin and glucose) one colony containing pSE100 was grown over nigth and infected with the helper phage R408 for production of an enriched phage stock. The resulting phage stock containing recombinant phages expressing the insert of pSE100 had a titer of 3x10^{9}cfu/ml. The phage stock of pSE100 was used to pan against 13 different proteins coated in microtiter wells and to one uncoated well. To each well containing the respective protein (or to the uncoated well) 200µl of the phage stock of pSE100 was added. After panning for three hours at RT under gentle agitation the wells were washed extensively using PBST and a sample of the last wash was collected. The bound phages were eluted with Na-Citrate buffer pH 1.8. The eluted samples were immediately nouralized using 1M Tris-HCl pH 8.6. The eluted phages and the phages from the wash were allowed to separately infect *E. coli* TG1 cells and after infection the cells were plated on LA plates containing ampicillin and glucose. The plates were incubated over night at 37°C and the frequency of colonies was counted. The result of this experiment is presented in Table 2 which shows the fibrinogen binding specificity of the protein expressed by pSE100.

Table 2.

Ligand	Wash	Eluat pH 1.8

Fibrinogen	$1.1x10^4$ cfu/ml	$1.4x10^7$ cfu/ml
α_2M	$2.0x10^2$ cfu/ml	$2.0x10^3$ cfu/ml
BSA	$<10^4$ cfu/ml	$8.0x10^2$ cfu/ml
Collagen typeI	$6.0x10^2$ cfu/ml	$1.2x10^3$ cfu/ml
Elastin	$8.0x10^2$ cfu/ml	$5.2x10^3$ cfu/ml
Fibronectin	$6.0x10^2$ cfu/ml	$2.4x10^4$ cfu/ml
HSA	$8.0x10^2$ cfu/ml	$2.2x10^3$ cfu/ml
IgA	$6.0x10^4$ cfu/ml	$6.8x10^4$ cfu/ml
IgG	$4.0x10^2$ cfu/ml	$4.4x10^3$ cfu/ml
Lactoferrin	$6.0x10^4$ cfu/ml	$8.2x10^3$ cfu/ml
Pepsin	$1.8x10^2$ cfu/ml	$3.7x10^4$ cfu/ml
Transferrin	$2.0x10^4$ cfu/ml	$2.4x10^3$ cfu/ml
Vitronectin	$<10^2$ cfu/ml	$2.2x10^3$ cfu/ml
Plastic	$2.4x10^3$ cfu/ml	$9.0x10^3$ cfu/ml

(M) inhibition experiment. The pSE100 phage stock was diluted to a titer of ~$5x10^4$cfu/ml. Of this phage solution samples (180µl) was taken and separately incubated for one hour with different concentrations of fibrinogen, BSA or IgG before transferred to fibrinogen coated microtiter wells. After panning for three hours at RT under gentle agitation the wells were washed extensively using PBST. The bound phages were eluted with Na-Citrate buffer pH 1.8. The eluted samples were immediately neutralized using 1M Tris-HCl pH 8.6. The eluted phages were allowed to infect *E. coli* TG1 cells and after infection the cells were plated on LA plates containing ampicillin and glucose. The plates were incubated over night at 37°C and the frequency of colonies was counted. The result of this experiment is presented in Table 3 which shows that the binding to fibrinogen is inhibited by fibrinogen but not with the other tested proteins.

Table 3.

Conc. of different ligands (µg/ml)	Soluable ligands *Fibrinogen*	*BSA*	*IgG*
0	$7.6x10^4$ cfu/ml	$7.6x10^4$ cfu/ml	$7.6x10^4$ cfu/ml
0.1	$4.4x10^4$ cfu/ml	$7.0x10^4$ cfu/ml	$6.2x10^4$ cfu/ml
1	$3.6x10^4$ cfu/ml	$9.3x10^4$ cfu/ml	$9.0x10^4$ cfu/ml
10	$1.5x10^4$ cfu/ml	$6.3x10^4$ cfu/ml	$7.8x10^4$ cfu/ml

100	$3.8x10^3$ cfu/ml	$6.4x10^4$ cfu/ml	$7.3x10^4$ cfu/ml
1000	$3.0x10^3$ cfu/ml	$6.9x10^4$ cfu/ml	$7.6x10^4$ cfu/ml

Example 5. Western blot experiment.

E. coli cells of strain TG1 and MC1061 containing pSE100 were grown in LB (containing ampicillin and glucose) over night at 37°C. The next morning the cells were harvested by centrifugation, resuspended in LB (containing ampicillin, glucose and 0.1 M IPTG and further incubated at 37°C. Twelve hours later the cells were harvested by centrifugation and both the cells and the supernatant were taken care of. Four volumes of aceton were added to the supernatant and the resulting precipitate was collected by centrifugation, airdried and resuspended in icecold PBS. Prior to electrophoresis the cells and the precipitate from the supernatant were resuspended separately in a sample buffer containing 2.5%SDS and 5% beta-mercaptoethanol and boiled for two minutes. After denaturation the samples were analysed run under reducing conditions using the PHAST-system (Pharmacia) on a 8-25% gradient gel using SDS-buffer strips After the electrophoresis was completed a NC-filter previously soaked in PBS was put on the gel and the temperature raised to 45°C. After ~45 minutes the NC-filter was wetted with 1 ml PBS, gentle rewoved and placed in 15ml PBS containing 0.1% Tween 20 solution (PBST 0.1%) for 30 minutes in RT (under gentle agitation and with two changes of PBST 0.1% solution). After the last change of PBST 0.1% fibrinogen was added to a final conc. of 20ng/ml and the filter was incubated for four hours at RT under gentle agitation. The filter was subsequently washed for 3x10 minutes using PBST0.1% and HRP-conjugated rabbit anti-human fibrinogen antibodies (DAKO code A 080, diluted 1:500 in PBST 0.1%) were added and the filter was incubated for 1 hour at RT under gentle agitation. After washing the filter 3x10 minutes using PBST 0.1% the bound fibrinogen was visulized by transferring the filter to a solution containing a substrate for the horse radish peroxidase (6 ml 4 chloro-1-naphtol (3 mg/ml in metanol) + 25 ml PBS + 20 µl H_2O_2). The result showed that a fibrinogen binding protein was found in both types of samples (cells and growth media) in both *E. coli* cells harbouring pSE100, while no such protein was found in the

control cultur s of *E. coli* TG1 and MC1061. The fibrinog n binding protein expressed from the pSE100 was in the approximate size as aspected from the deduced amino acid.

Example 6 The occurrence of the *fig* gene and the use of *fig* gene to identify *S. epidermidis* in diagnostic test.

Purified chromosomal DNA from *S. aureus* strain 8325-4, *Streptococcus equi* subsp. *equi* strain 196 and subsp. *zooepidemicus* strain 25, *Streptococcus pyogenes* strain 2-1047, *Streptococcus dysgalactiae* strain 8215 were cleaved using the restriction enzyme *Eco*RI. The cleaved samples were run on an 0.8% agarose-gel together with chromosomal DNA from *S. epidermidis* strain HB cleaved with various restriction enzymes. After the electrophoresis was completed the separated DNA fragments were transferred to a NC-filter using the Vaccum blotting system from Pharmacia. After the transfer the filter was hybridized under stringent conditions(in a solution containing 6xSSC, 5xDenhart, 0.5%SDS at 65°C) using a probe designed on the basis of the nucleotide sequence of the insert of pSE100. This probe had earlier been prepared as follows, two oligonucliotides (5′-AGGTCAAGGACAAGGTGAC-3′and 5′-CAACAACCATCTCACACAAC-3′) were ordered (Pharmacia) and used as a primer pair in a PCR (25 cycles of 94°C 1 minute, 50°C 30 seconds, 72°C 1 minute using an Perkin Elmer Cetus Thermal Cycler 480) to amplify an ~150 bp fragment of the insert of pSE100. The amplified material was run on an agarose gel and the ~150 bp fragment was purified and radioactively labelled using α^{32}P-dATP and the Multiprime DNA labelling system (Amersham). The filter was hybridized over night and subsequently washed in a washing solution (0.2% SSC, 0.1% SDS) at 60°C and autoradiographed. The result showed that no hybridization was detected in the samples originating from streptococci and *S. aureus* while hybridization occurred to the samples comming from the *S. epidermidis* strain HB.

To investigate the occurrence of the *fig* gene in other strains of *S. epidermidis* the following PCR reaction was set up. Chromosomal DNA from 13 different clinial isolates of *S. epidermidis* were used as templates. The sam primers and the same PCR conditions as described above were used. The result showed that an amplified

product of ~150 bp could b detected (using a 2% agarose gel) in all strains of *S. epidermidis* but not in the control samples original containing chromosomal DNA from *S. aureus* and *S. pyogenes*.

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WO 84/03103

Sequenc list

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          10         20         30         40         50         60         70
ACCACCACCACCACCACCACCCCTCTAGTGATGAAGAAAAGAATGATGTGATCAATAATAATCAGTCAATAA
   H  H  H  H  H  H  P  S  S  D  E  E  K  N  D  V  I  N  N  N  Q  S  I
<-- Pel leader

          80         90        100        110        120        130        140
ACACCGACGATAATAACCAAATAATTAAAAAAGAAGAAACGAATAACTACGATGGCATAGAAAAACGCTCAG
N  T  D  D  N  N  Q  I  I  K  K  E  E  T  N  N  Y  D  G  I  E  K  R  S

         150        160        170        180        190        200        210
AAGATAGAACAGAGTCNACAACAAATGTNGATGAAAACGAAGCAACATTTTTACAAAAGACCCCTCAAGATA
E  D  R  T  E  X  T  T  N  X  D  E  N  E  A  T  F  L  Q  K  T  P  Q  D

         220        230        240        250        260        270        280
ATACTCATCTTACAGAAGAAGAGGTNAAAGAATCCTCATCAGTCGAATCCTCAAATTCATCAATTGATACTG
N  T  H  L  T  E  E  E  X  K  E  S  S  S  V  E  S  S  N  S  S  I  D  T

290        300        310        320        330        340        350        360
CCCAACAACCATCTCACACAACAATAAATAGAGAAGAATCTGTTCAAACAAGTGATAATGTAGAAGATTCAC
A  Q  Q  P  S  H  T  T  I  N  R  E  E  S  V  Q  T  S  D  N  V  E  D  S

         370        380        390        400        410        420        430
ACGTATCAGATTTTGCTAACTCTAAAATAAAAGAGAGTAACACTGAATCTGGTAAAGAAGAGAATACTATAG
H  V  S  D  F  A  N  S  K  I  K  E  S  N  T  E  S  G  K  E  E  N  T  I

         440        450        460        470        480        490        500
AGCAACCTAATAAAGTAAAAGAAGATTCAACAACAAGTCAGCCGTCTGGCTATACAAATATAGATGAAAAAA
E  Q  P  N  K  V  K  E  D  S  T  T  S  Q  P  S  G  Y  T  N  I  D  E  K
```

Sequence list cont.

```
510 520 530 540 550 560 570
TTTCAAATCAAGATGAGTTATTAAATTTACCAATAAATGAATATGAAAATAAGGCTAGACCATTATCTACAA
I S N Q D E L L N L P I N E Y E N K A R P L S T

580 590 600 610 620 630 640
CATCTGCCCAACCATCGATTAAACGTGTAACCGTAAATCAATTAGCGGCGGAACAACGTTCGAATGTTAACC
T S A Q P S I K R V T V N Q L A A E Q G S N V N

650 660 670 680 690 700 710 720
ATTTAATTAAAGTTACTGATCAAAGTATTACTGAAGGATATGATGATAGTGAAGGTGTTATTAAAGCACATG
H L I K V T D Q S I T E G Y D D S E G V I K A H

730 740 750 760 770 780 790
ATGCTGAAAACTTAATCTATGATGTAACTTTTGAAGTAGATGATAAGGTGAAATCTGGTGATACGATGACAG
D A E N L I Y D V T F E V D D K V K S G D T M T

800 810 820 830 840 850 860
TGGATATAGATAAGAATACAGTTCCATCAGATTTAACCGATAGCTTTACAATACCAAAAATAAAAGATAATT
V D I D K N T V P S D L T D S F T I P K I K D N

870 880 890 900 910 920 930
CTGGAGAAATCATCGCTACAGGTACTTATGATAACAAAAATAAACAAATCACCTATACTTTTACAGATTATG
S G E I I A T G T Y D N K N K Q I T Y T F T D Y

940 950 960 970 980 990 1000
TAGATAAGTATGAAAATATTAAAGCACACCTTAAATTAACGTCATACATTGATAAATCAAAGGTTCCAAATA
V D K Y E N I K A H L K L T S Y I D K S K V P N

1010 1020 1030 1040 1050 1060 1070 1080
ATAATACCAAGTTAGATGTAGAATATAAAACGGCCCTTTCATCAGTAAATAAAACAATTACGGTTGAATATC
N N T K L D V E Y K T A L S S V N K T I T V E Y

1090 1100 1110 1120 1130 1140 1150
AAAGACCTAACGAAAATCGGACTGCTAACNTTCAAAGTATGTTTACAAATATAGATACCAAAAATCATACAG
Q R P N E N R T A N X Q S M F T N I D T K N H T

1160 1170 1180 1190 1200 1210 1220
TTGAGCAAACGATTTATATTAACCNTCTTCGTTATTCAGCCAAGGAAACAAATGTAAATATTTCAGGGAAT
V E Q T I Y I N X L R Y S A K E T N V N I S G N
```

Sequ nce list cont.

```
 1230      1240      1250      1260      1270      1280      1290
GTGATGAAGGTTCAACAATTATAGACGATAGCACAATAATTAAAGTTTATAAGGTTGGAGATAATCAAAATT
G  D  E  G  S  T  I  I  D  D  S  T  I  I  K  V  Y  K  V  G  D  N  Q  N

1300      1310      1320      1330      1340      1350      1360
TACCAGATAGTAACAGAATTTATGATTACAGTGAATATGAAGATGTCACAAATGATGATTATGCCCAATTAG
L  P  D  S  N  R  I  Y  D  Y  S  E  Y  E  D  V  T  N  D  D  Y  A  Q  L

1370      1380      1390      1400      1410      1420      1430      1440
GAAATAATAATGATGTGAATATTAATTTTGGTAATATAGATTCACCATATATTATTAAAGTTATTAGTAAAT
G  N  N  N  D  V  N  I  N  F  G  N  I  D  S  P  Y  I  I  K  V  I  S  K

     1450      1460      1470      1480      1490      1500      1510
ATGACCNTAATAAGGATGATTACACGACTATACAGCAAACTGTGACAATGCAGACGACTATAAATGAGTATA
Y  D  X  N  K  D  D  Y  T  T  I  Q  Q  T  V  T  M  Q  T  T  I  N  E  Y

    1520      1530      1540      1550      1560      1570      1580
CTGGTGAGTTTAGAACAGCATCCTATGATAATACAATTGCTTTCTCTACAAGTTCAGGTCAAGGACAAGGTG
T  G  E  F  R  T  A  S  Y  D  N  T  I  A  F  S  T  S  S  G  Q  G  Q  G

 1590      1600      1610      1620      1630      1640      1650
ACTTGCCTCCTGAAAAAACTTATAAAATCGGAGATTACGTATGGGAAGATGTAGATAAAGATGGTATTCAAA
D  L  .  P  E  K  T  Y  K  I  G  D  Y  V  W  E  D  V  D  K  D  G  I  Q

1660      1670      1680      1690      1700      1710      1720
ATACAAATGATAATGAAAAACCGCTTAGTAATGTATTGGTAACTTTGACGTATCCTGATGGAACTTCAAAAT
N  T  N  D  N  E  K  P  L  S  N  V  L  V  T  L  T  Y  P  D  G  T  S  K

1730      1740      1750      1760      1770      1780
CAGTCAGAACAGATGAAGATGGGAAATATCAATTTGATGGGGTGCAGGTCGAC
S  V  R  T  D  E  D  G  K  Y  Q  F  D  G  V  Q  V  D
                                          ------------>
                                          Myc tail
```

Sequence list. A partial nucleotide sequence of the putative *fig* gene from *S. epidermidis* strain HB and the deduced amino acid sequence. The vector s qu nces in the junction of the 5′- and 3′-ends are indicated.

Claims

1. Recombinant DNA molecule containing a nucleotide sequence coding for a protein or polypeptide having fibrinogen binding activity originating from a coagulase-negative staphylococcal strain.

2. Plasmid, phage or phagemid containing a nucleotide sequence coding for a protein or polypeptide having fibrinogen binding activity originating from a coagulase-negative staphylococcal strain.

3. Microorganism containing at least one recombinant DNA molecule according to claim 1.

4. Microorganism containing at least one plasmid, phage or phagemid according to claim 2.

5. Method for producing a fibrinogen binding protein or a polypeptide thereof, characterized in that
- at least one recombinant DNA molecule according to claim 1 is introduced in a microorganism,
- said micrcorganism is cultured in a suitable medium,
- the protein thus formed is isolated by chromatographic purification.

6. Method for producing a fibrinogen binding protein or polypeptide thereof characterized in that
- at least one recombinant protein according to claim 1 is expressed on a phage particle,
- said phage particle shows fibrinogen binding activity

7. Recombinant DNA molecule according t claim 1, charact rized by containing one or more of th following nucleotid sequ nces:

```
                10        20        30        40        50        60        70
                |         |         |         |         |         |         |
   1 CTCTAGTGATGAAGAAAAGAATGATGTGATCAATAATAATCAGTCAATAAACACCGACGATAATAACCAA
  71 ATAATTAAAAAAAGAAGAAACGAATAACTACGATGGCATAGAAAAACGCTCAGAAGATAGAACAGAGTCNA
 141 CAACAAATGTNGATGAAAACGAAGCAACATTTTTACAAAAGACCCCTCAAGATAATACTCATCTTACAGA
 211 AGAAGAGGTNAAAGAATCCTCATCAGTCGAATCCTCAAATTCATCAATTGATACTGCCCAACAACCATCT
 281 CACACAACAATAAATAGAGAAGAATCTGTTCAAACAAGTCATAATGTAGAAGATTCACACGTATCAGATT
 351 TTGCTAACTCTAAAATAAAAGAGAGTAACACTGAATCTGGTAAAGAAGAGAATACTATAGAGCAACCTAA
 421 TAAAGTAAAAGAAGATTCAACAACAAGTCAGCCGTCTGGCTATACAAATATAGATGAAAAAATTTCAAAT
 491 CAAGATGAGTTATTAAATTTACCAATAAATGAATATGAAAATAAGGCTAGACCATTATCTACAACATCTG
 561 CCCAACCATCGATTAAACGTGTAACCGTAAATCAATTAGCGGCGGAACAAGGTTCGAATGTTAACCATTT
 631 AATTAAAGTTACTGATCAAAGTATTACTGAAGGATATGATGATAGTGAAGGTGTTATTAAAGCACATGAT
 701 GCTGAAAACTTAATCTATGATGTAACTTTTGAAGTAGATGATAAGGTGAAATCTGGTGATACGATGACAG
 771 TGGATATAGATAAGAATACAGTTCCATCAGATTTAACCGATAGCTTTACAATACCAAAAATAAAAGATAA
 841 TTCTGGAGAAATCATCGCTACAGGTACTTATGATAACAAAAATAAACAAATCACCTATACTTTTACAGAT
 911 TATGTAGATAAGTATGAAAATATTAAAGCACACCTTAAATTAACGTCATACATTGATAAATCAAAGGTTC
 981 CAAATAATAATACCAAGTTAGATGTAGAATATAAAACGGCCCTTTCATCACTAAATAAAACAATTACGGT
1051 TGAATATCAAAGACCTAACGAAAATCGGACTGCTAACNTTCAAAGTATGTTTACAAATATAGATACGAAA
1121 AATCATACAGTTGAGCAAACGATTTATATTAACCNTCTTCGTTATTCAGCCAAGGAAACAAATGTAAATA
1191 TTTCAGGGAATGGTGATGAAGGTTCAACAATTATAGACGATAGCACAATAATTAAAGTTTATAAGGTTGG
1261 AGATAATCAAAATTTACCAGATAGTAACAGAATTTATGATTACAGTGAATATGAAGATGTCACAAATGAT
1331 GATTATGCCCAATTAGGAAATAATAATGATGTGAATATTAATTTTGGTAATATAGATTCACCATATATTA
1401 TTAAAGTTATTAGTAAATATGACCNTAATAAGGATGATTACACGACTATACAGCAAACTGTGACAATGCA
1471 GACGACTATAAATGAGTATACTGGTGAGTTTAGAACAGCATCCTATGATAATACAATTGCTTTCTCTACA
1541 AGTTCAGGTCAAGGACAAGGTGACTTGCCTCCTGAAAAAACTTATAAAATCGGAGATTACGTATGGGAAG
1611 ATGTAGATAAAGATGGTATTCAAAATACAAATGATAATGAAAAACCGCTTAGTAATGTATTGGTAACTTT
1681 GACGTATCCTGATCGAACTTCAAAATCAGTCAGAACAGATGAAGATGGGAAATATCAATTTGATG
```

8. Recombinant DNA molecule according to claim 1, characterized by encoding one or more of the following amino acid sequences:

```
                5                  10                  15                  20                  25                  30
                |                  |                   |                   |                   |                   |
  1 S S D E E K N D V I N N N Q S I N T D D N N Q I I K K E E T
 31 N N Y D G I E K R S E D R T E X T T N X D E N E A T F L Q K
 61 T P Q D N T H L T E E E X K E S S S V E S S N S S I D T A Q
 91 Q P S H T T I N R E E S V Q T S D N V E D S H V S D F A N S
121 K I K E S N T E S G K E E N T I E Q P N K V K E D S T T S Q
151 P S G Y T N I D E K I S N Q D E L L N L P I N E Y E N K A R
181 P L S T T S A Q P S I K R V T V N Q L A A E Q G S N V N H L
211 I K V T D Q S I T E G Y D D S E G V I K A H D A E N L I Y D
241 V T F E V D D K V K S G D T M T V D I D K N T V P S D L T D
271 S F T I P K I K D N S G E I I A T G T Y D N K N K Q I T Y T
301 F T D Y V D K Y E N I K A H L K L T S Y I D K S K V P N N N
331 T K L D V E Y K T A L S S V N K T I T V E Y Q R P N E N R T
361 A N X Q S M F T N I D T K N H T V E Q T I Y I N X L R Y S A
391 K E T N V N I S G N G D E G S T I I D D S T I I K V Y K V G
421 D N Q N L P D S N R I Y D Y S E Y E D V T N D D Y A Q L G N
451 N N D V N I N F G N I D S P Y I I K V I S K Y D X N K D D Y
481 T T I Q Q T V T M G T T I N E Y T G E F R T A S Y D N T I A
511 F S T S S G Q G Q G D L P P E K T Y K I G D Y V W E D V D K
541 D G I Q N T N D N E K P L S N V L V T L T Y P D G T S K S V
571 R T D E D G K Y Q F D
```


9. Plasmid, phage or phagemid containing one or more nucleotide sequences according to claim 7.

10. Microorganism containing at least one plasmid, phage or phagemid according to claim 8.

11. The use of an extractable fraction of coagulase-negative staphylococci to block the adherence of coagulase-negative staphylococci to surfaces with immobilized fibrinogen.

12. The use of the native fibrinogen binding protein or parts thereof from coagulase-negative staphylococci to block the adherence of coagulase-negative staphylococci to surfaces with immobilized fibrinogen.

13. The use of protein FIG or parts thereof to block the adherence of coagulase-negative staphylococci to surfaces.

14. The use of immobilized protein FIG or fragments thereof to isolate or detect fibrinogen in solutions.

15. For diagnostic purposes use the *fig* gene or parts thereof to detect the presence of *S. epidermidis*.

16. The use of antibodies against the extractable fraction of coagulase-negative staphylococci to block the adherence of coagulase-negative staphylococci.

17. The use of antibodies against the native fibrinogen binding protein from coagulase-negative staphylococci to block the adherence of coagulase-negative staphylococci.

18. The use of antibodies against protein FIG or parts thereof to block the adherence of coagulase-negative staphylococci.

19. The use of a fibrinogen binding protein or parts thereof from coagulase-negative staphylococci as an immunogen.

20. The use of a protein FIG or parts thereof as an immunogen.

Abstract

Methods for producing a protein or polypeptide having fibrinogen binding activity and a recombinant DNA molecule coding for said protein (or fragments thereof), and for microorganisms (including virus) containing this recombinant DNA molecule.

Figure belongs to Swedish patent ansökan
Method and Means for producing a fibrinogen binding Protein
and its use i Bioteknologi 960620
Sökande: Bengt Guss etal

Ink. t. Patent- och reg.verket
1996 -06- 20
Huvudfaxen Kassan

Fig 1

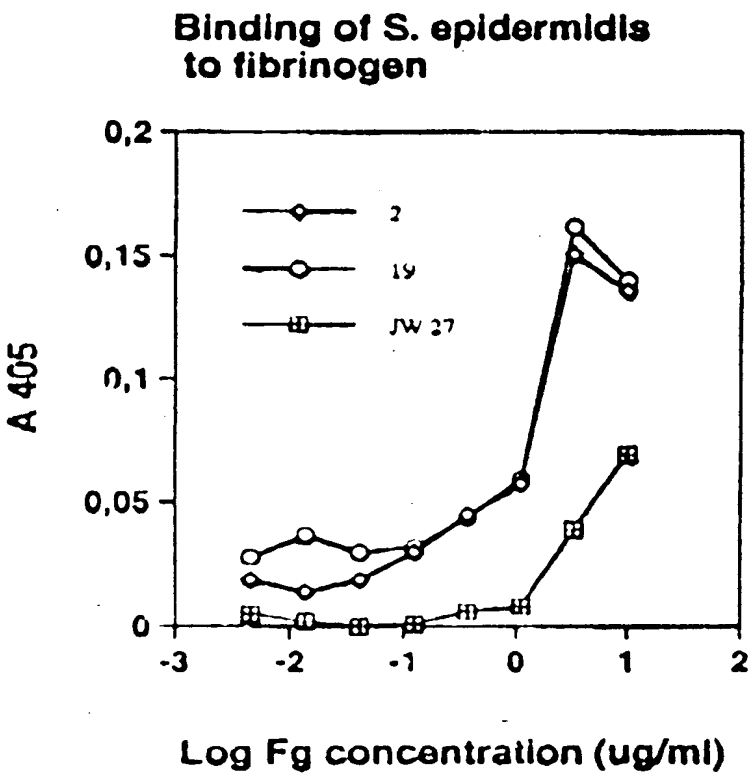

Figure 2

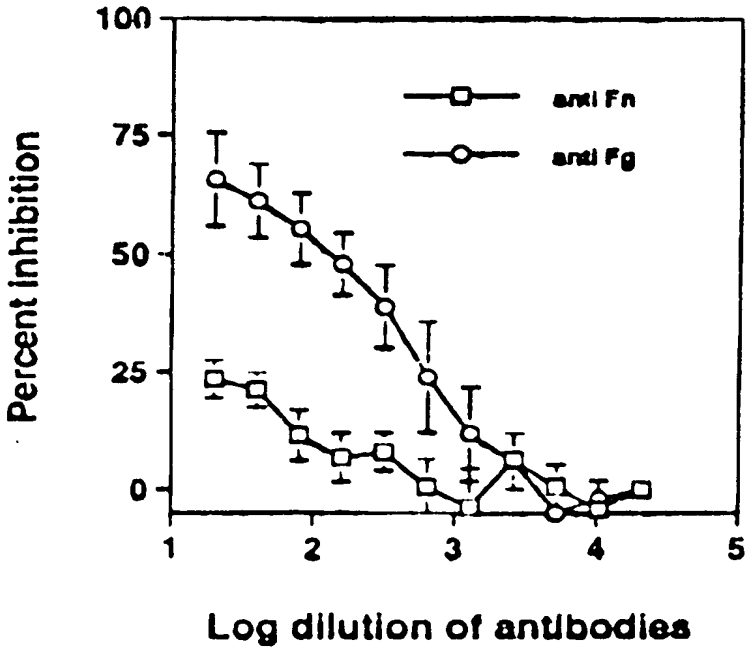

Figure 3

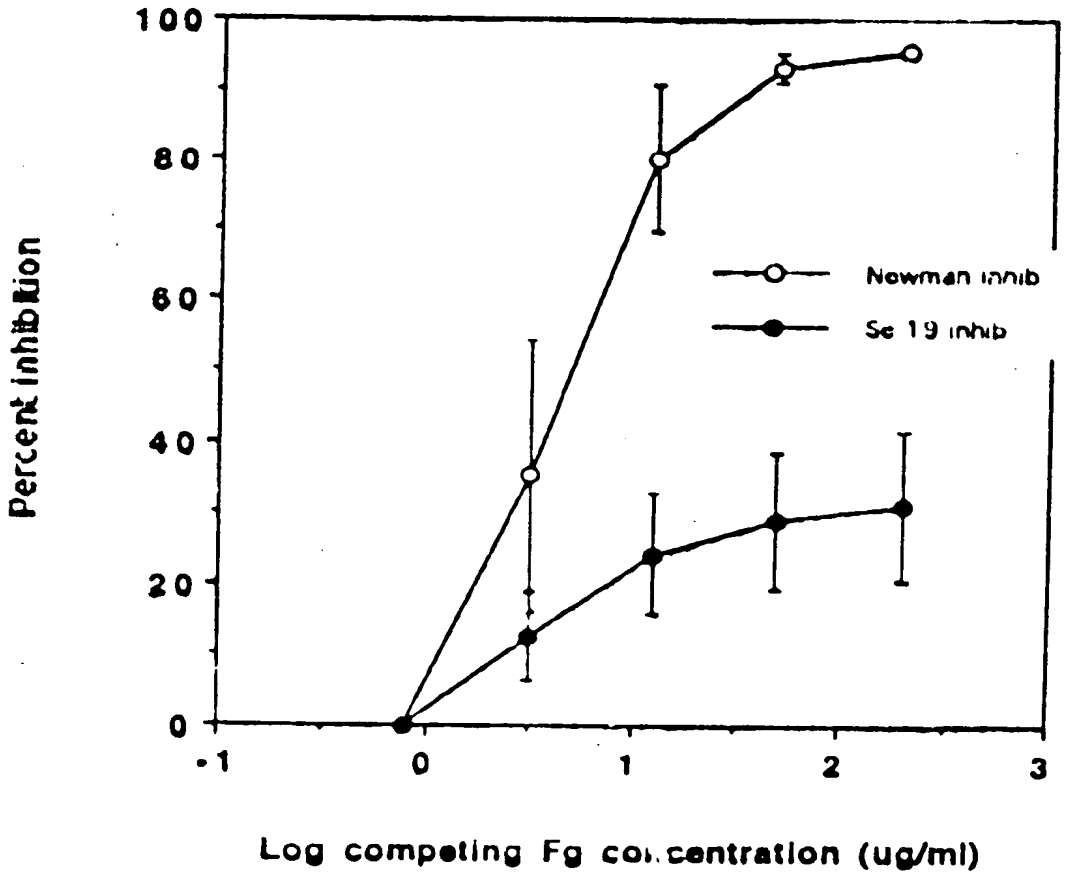

Figure 4

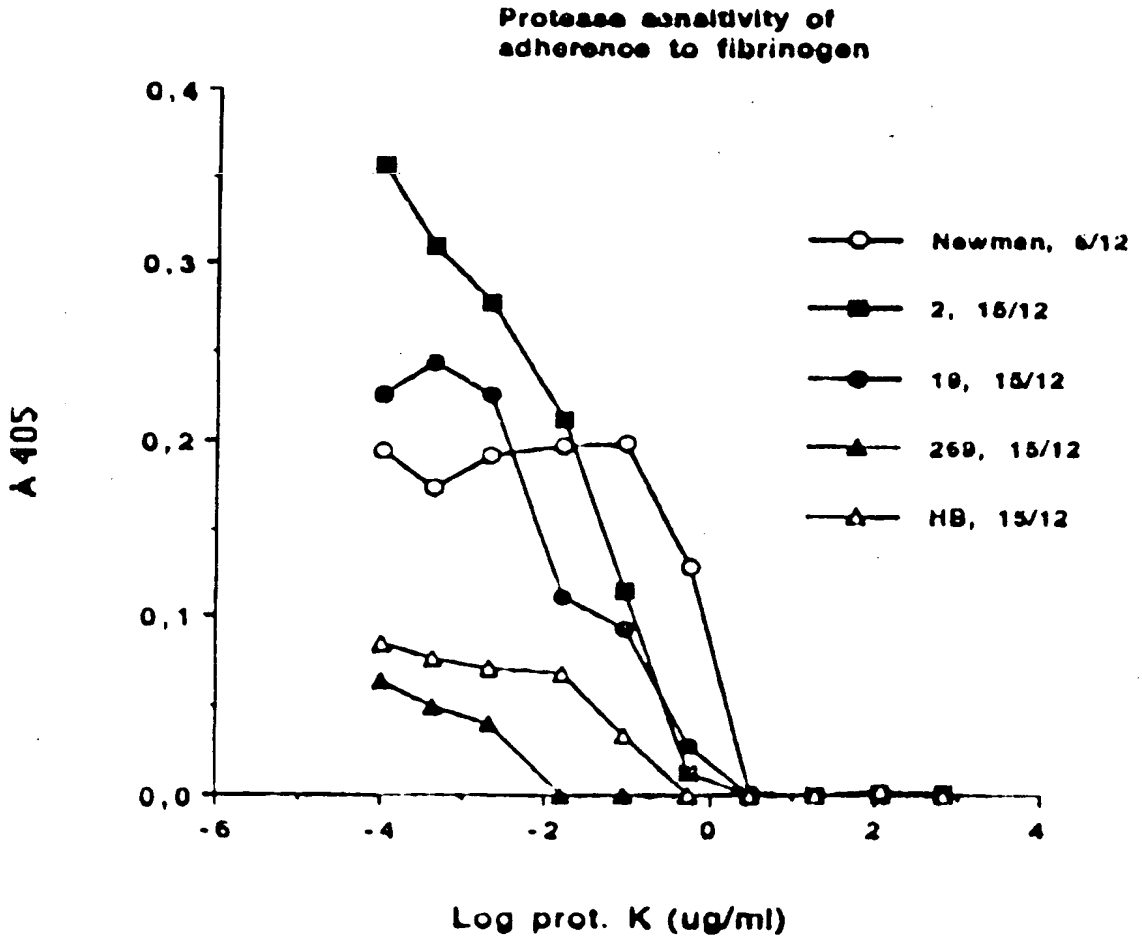

Figure 5